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AWARD NUMBER: DAMD17-01-1-0342

TITLE: Susceptibility of BRCA2 Heterozygous Normal Mammary Epithelial Cells to Radiation-Induced Transformation

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REPORT DATE: October 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-10-2005	Annual	17 Sep 2004 – 16 Sep 2005

4. TITLE AND SUBTITLE

Susceptibility of BRCA2 Heterozygous Normal Mammary Epithelial Cells to Radiation-Induced Transformation

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-01-1-0342

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Qingshen Gao, M.D.

E-Mail:

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Evanston Northwestern Healthcare Research Institute
Evanston, Illinois 02111

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

In the approved Statement of Work, we proposed six tasks for the first two years. Task3 turned out to be much more difficult than we have anticipated. Thus far we have not yet obtained clones with one allele of BRCA2 disrupted even after numerous attempts. In last year's reported we proposed three alternative strategies. We have performed all three sets of experiments. We constructed the promoter less constructs to target exon11. We also constructed the promoter less constructs to target exon2. However, we have failed to obtain BRCA2+/- celllines. Our alternative strategy, the RNAi strategy has been successful. We already obtained stable celllines with reduced levels of BRCA2 expression. Since RNAi does not ideally mimic the in vivo situation, we will use these celllines as a backup. We have preliminary data indicated that cell surface marker such as CD19 and Hook-2 can be used as efficient selection markers using magnetic beads. We are currently in the process of generating knock-out construct s using CD19 and Hook-2 as selection markers. OnceBRCA2+/-celllines are generated, the remaining task of the project will be carried out.

15. SUBJECT TERMS
BRCA2, radiation, transformation, immortalization, breast cancer, tumor suppressor gene

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	28	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Cover……………………………………………………………………………………1

SF 298……………………………………………………………………………..……2

Table of Contents...……………………………………………………….………....3

Introduction…………………………………………………………….……….....4

Body………………………………………………………………………………….8

Key Research Accomplishments………………………………………….………11

Reportable Outcomes……………………………………………………………….11

Conclusions…………………………………………………………………………..11

References…………………………………………………………………………….25

INTRODUCTION

Breast cancer is by far the most frequently diagnosed cancer in women. Each year over 186,000 new cases and 46,000 deaths are reported in United State alone(1). Germ-line mutations in the breast cancer susceptibility genes BRCA2 and BRCA1 confer susceptibility to familial early-onset breast and ovarian cancers (2) (3-6). So far very few somatic mutations of BRCA2 have been found in sporadic breast cancer. However, loss of heterozygosity at the BRCA2 locus has been observed in 30-40% of sporadic breast tumors (7-12). It has been noted that the genomic region of BRCA1 and BRCA2 genes contains very high densities of repetitive DNA elements, which can lead to high genomic instability (13). This distinctive genomic instability might contribute to the inactivation of BRCA2 in sporadic breast or ovarian cancer (13).

Extensive studies indicated that BRCA2 plays an important role in DNA repair. Several studies found that BRCA2 binds to BRCA1 and Rad51 and co-localizes with them in the nucleus (14-17). Rad51 is a homologue of the *Escherichia coli recA* gene. Rad51 is known to function in recombinational repair of double strand DNA breaks (18-29). Binding of BRCA2 to Rad51 suggests that BRCA2 and Rad51 might function in the same pathway. Studies with BRCA2 knock out mice further confirmed the role of BRCA2 in DNA repair. Sharan et al first reported that BRCA2 null mouse embryos are hypersensitive to γ-radiation (14). These data were further confirmed with embryonic stem (ES) cells and mouse embryonic fibroblasts (MEF) with less severe BRCA2 truncations (22-24). Particularly, spontaneous accumulation of chromosomal abnormalities, including breaks and aberrant chromatid exchanges was observed in BRCA2 null MEFs(23). The human pancreatic tumor cell line, Capan-1, was found to have the 6174delT mutation in its BRCA2 gene and loss of the other allele (25-27). In correlation with the observation in BRCA2 knock-out MEFs, Capan-1 cells are extremely sensitive to ionizing radiation and DNA damaging agents (26-27). Importantly, reconstituted expression of wild-type BRCA2 in Capan-1 cells confers to these cells resistance to DNA damaging agents (27). Furthermore, antisense BRCA2 deoxyribonucleotides that can decrease the expression of BRCA2 RNA in cells significantly increase the sensitivity of these cells to DNA damaging agents (26). Additionally, overexpression of a BRC repeat in MCF-7 cells ($BRCA2^{+/+}$) that attenuates the function of BRCA2 rendered these cells sensitive to DNA damaging agents, and abrogated the G2-M checkpoint induced by DNA damage (28). Recent studies directly demonstrated that BRCA2 plays a role the homology-directed recombinational repair of double strand DNA breaks (29-32). Two structural studies provide further confirmation for the role of BRCA2 in the homology-directed recombinational repair of double strand DNA breaks (33-35). These data clearly underscore the roles of BRCA2 in DNA damage response in human cells.

In addition to surgery and systemic therapy, radiation therapy is one of the most important components of breast cancer management. Lumpectomy and radiation therapy (LRT) remains a standard of care option for the majority of patients with early stage breast cancer. It is estimated that about 40-60% breast cancer patients with early stage disease will undergo radiation treatment (36-38). However, development of radiation-

induced secondary malignancy after successful radiotherapy of a prior cancer has been well documented. It is generally accepted among radiation oncologists that for most patients this is an unavoidable complication (39). However, for a specific population of patients, such as BRCA2 or BRCA1 germline mutation carriers, the risk of radiation induced breast cancer warrants further investigation.

Since BRCA2 has been proven to play a important role in the double stranded DNA damage repair, breast cancer patients with a germline BRCA2 mutation may be more susceptible to radiation induced transformation. First, this could be due to haploinsufficiency. Although the classic Knudson's two-hit model of tumorigenesis stipulates that mutation of both alleles of a tumor suppressor gene is needed to trigger tumor formation, a number of studies indicated that mutation or loss of a single allele may be sufficient to exert a cellular phenotype that leads to tumorigenesis without inactivation of the second allele. Recently, haploinsufficiency of BLM gene, another tumor suppressor gene product that is involved in DNA repair, have been demonstrated to enhance tumorigenesis. It has been a puzzling observation that while loss of heterozygosity at the BRCA2 locus has been detected in 30-40% of sporadic breast and ovarian tumors, very few mutations were ever found in these sporadic cases (8-12, 40). These studies imply that deletion of one copy of BRCA2 can also compromise some functions of BRCA2 and permit tumorigenesis. Notably, in mouse knock out system, there is no statistically significant difference observed between BRCA2 wild type and BRCA2 heterozygous embryos or cells in their sensitivity to DNA damaging agents (14, 22-24). However, the response of human mammary epithelial cells could be very different from mouse ES cells or MEFs, and should be directly assessed. Furthermore, it should be noted that the sensitivity to radiation is different from the susceptibility to radiation-induced transformation. A slight increase in radiation sensitivity, which may escape from detection, could lead to more mutations to be accumulated than a more significant increase in radiation sensitivity, which usually eliminated the mutated cells. Interestingly, Patel et al consistently observed a small difference between wild-type and heterozygous cells in their sensitivity to DNA damaging agents (23). Also, even without haploinsufficiency, because the BRCA2 gene occupies a large locus, cells with only one copy of BRCA2 will be more vulnerable to inactivation by radiation. Once the only wild type copy of BRCA2 gene is inactivated by radiation, the DNA repair will be impaired, further mutations will accumulate, leading to transformation.

To test this hypothesis that **radiation treatment of breast cancer patients who inherit one defective copy of the BRCA2 gene may pose greater risk of radiation-induced secondary cancer**, we propose to generate BRCA2 $^{+/-}$ cells from normal human mammary epithelial cells, and directly compare the susceptibility of the BRCA2 $^{+/-}$ cells and their parental cells to radiation induced transformation.

Clinical study would have been a good system to test this hypothesis, however, it is hindered by small numbers of patients with documented BRCA2 status, long term follow up, and interference of multigenic effects.

Knock out mouse systems has very limit value. The studies that are carried out in mouse knock out system, especially in fibroblasts of mouse origin may not be correct in human mammary epithelial system. Mouse cells are very susceptible to transformation by chemical carcinogens, radiation, tumor viruses or oncogenes. Spontaneous immortalization and transformation can be obtained easily. In contrast, human cells are very resistant to transformation by all the transforming agents. Spontaneous immortalization almost never occurs in human cell culture system (41-42). Also, BRCA2 is poorly conserved from mouse to human. Comparison of the amino acids sequence of human BRCA2 and mouse BRCA2 indicated that human BRCA2 exhibits only 59% identity with mouse BRCA2, while the other tumor suppressor genes, such as APC, WT1, NF1, and RB1 exhibit greater than 90% identity (43).

One pair of normal human mammary epithelial cell strains with identical genetic background and different BRCA2 status will be an ideal system to assess the susceptibility of BRCA2 heterozygous normal human mammary epithelial cells to radiation induced transformation. The susceptibility to radiation-induced transformation can be affected by many genetic factors. Knocking out one copy of BRCA2 from a mammary epithelial cell strain with normal BRCA2 genotype will generate a normal mammary epithelial cell strain with identical genetic background to its parental strain with the exception of being BRCA2 heterozygous. These BRCA2$^{+/-}$ cells and their parental cells will be an ideal system to study the effects of being BRCA2 heterozygous on the susceptibility to radiation-induced transformation (Fig. 1). As the parental cells are very resistant to radiation induced transformation (unpublished observation), the effects of being BRCA2 heterozygous will be easily assessed. In this application we proposed to generate a BRCA2 heterozygous mammary epithelial cell strain, and directly compare the susceptibility of the BRCA2$^{+/-}$ cells and its parental cells to radiation-induced transformation (see Fig 1).

Somatic cells knock out technology is the most direct and unambiguous way to eliminate the function of a gene. In spite of many recent advances, somatic cells knock out remains a very difficult and arduous approach, which is still considered complex and high-risk. The reason for this is that in mammalian cells nonhomologous recombination is orders of magnitude more frequent than homologous recombination. Two approaches have been developed to enrich the homologous recombination (44, 45). The first approach is the "positive-negative" selection method. The negative selection can prevent non-homologous recombinants from scoring as colonies. The second approach is the so called promoterless approach, in which the expression of resistance gene is conditional on recombination at the homologous target site. We proposed the "positive-negative" method in our original proposal because more successful reports were published. We also planned the promoterless method because more successes were reported recently.

In the approved Statement of Work, we proposed three tasks for the first two years:

Task 1. Generate the BRCA2 Knock out construct and the positive control template constructs, sequence the construct. (months1-6)

Task 2. Confirm the status of BRCA2 is normal in normal human mammary epithelial cells. (months1-3)

Task 3. Transfect the knock out construct into normal mammary epithelial cells and screen for clones with one allele of BRCA2 disrupted. (months7-12)

Task 4. Assess susceptibility of BRCA2$^{+/-}$ human mammary epithelial cells to spontaneous immortalization by sub-culturing. (months 13-20)

Task 5. Characterize the spontaneous immortalized cells. (months 20-26)

Task 6. Assess susceptibility of BRCA2$^{+/-}$ human mammary epithelial cells to radiation induced immortalization. (months 13-24)

We have successfully accomplished task 1-2 in the first year. Task 3 turned out to be much more difficult than we have anticipated. We modified our approaches last year. We attempted three alternative approaches. We generated one set of constructs using promoterless selection targeting exon 11 (Table 1, Fig. 3), another set of promoterless vectors targeting exon 2 (Table 1, Fig. 4). Thus far we have not yet obtained clones with one allele of BRCA2 disrupted after numerous attempts. We also attempted the RNAi approaches (Table 1, Fig. 7). Stable cell clones with BRCA2 expression level reduced to different levels were successfully obtained. Since RNAi approach does not mimic ideally the in vivo situation. We will use it as a backup. We are currently in the process of more attempts with two more modified strategies (Table 1).

Additionally, The PI moved from New England Medical Center Tufts University to Evanston Northwestern Institute, Northwestern University School of Medicine on August 1, 2003. The funding has yet been transferred to the new institute. During this transition period, the progress of this project was not carried out at normal speed because of the move and the personal changes.

REPORT BODY

Task 1: Generate the BRCA2 Knock out construct and the positive control template construct, and sequence the constructs. (months1-6).

Completed in the first year.

Task 2: Characterization of BRCA2 gene in a normal human mammary epithelial cell strain. (months1-3)

Completed in the first year.

Task 3. Transfect the knock out construct into normal mammary epithelial cells and screen for clones with one allele of BRCA2 disrupted. (months7-12)

After numerous attempts, our original approach to use positive and negative selection vector to generate a normal mammary epithelial cell line with one allele of BRCA2 disrupted was not successful (Table 1, Fig. 2). We have explored a number of new approaches to circumvent this problem. We modified the original positive-negative selection construct into a promoterless knock out construct configuration (Table 1, Fig. 3). We also constructed another set of promoterless construct to target exon 2 (Table 1, Fig. 3). However, so far we have unsuccessful in obtaining mammary epithelial cell line with one allele of BRCA2 disrupted. We also explored the use GFP as a selection marker using FACS sorting. Unfortunately, these cells are unable to sustain FACS sorting. On other hand our RNAi strategy is successful. We already obtained a number of clones with BRCA2 expression level reduced to different levels, which are suitable for assessing their susceptibility to radiation induced transformation. However, RNAi strategy does not ideally mimic the in vivo situation. We plan to use it as a backup strategy. We found that we can efficiently select the normal mammary epithelial cells expressing a surface marker using magnetic beads. We proposed to use two kind of surface markers, CD19 and Hook-2 for selecting the correctly recombinated clones.

A. Transfect the knock out construct into normal mammary epithelial cells and screen for clones with one allele of BRCA2 disrupted.

Experiments were completed in the first year; no positive clone was obtained (Table 1, Fig. 2).

B. Transfect the knock out construct into hTERT immortalized normal mammary epithelial cells and screen for clones with one allele of BRCA2 disrupted.

Experiments were completed in the first year, no positive clone was obtained. We repeated this experiment again and we did not obtained any positive clones. These failures prompt us to improve our knock out constructs as described in the following.

C. Transfect the promoterless knock out construct into hTERT immortalized normal mammary epithelial cells and screen for clones with one allele of BRCA2 disrupted. Although the positive-negative selection knock out constructs has been used successfully in many cases, it was reported that the promoterless knock out construct configuration provide much better chances of success (44). To construct the promoterless knock out construct, we cloned the selection marker genes of Neomycin and Puromycin from pIRESneo and pIRESpuro into pKO-NYKV-BRCA2 to replace the original Neo expression cassette (Fig. 3a). One set of positive control constructs were also generated (Fig. 3b). The pIRESpuro or pIRESneo vectors contain the internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV), which permits the translation of two open reading frames from one messenger RNA. The ECMV IRES followed by the gene encoding puromycin resistance (puromycin-N-acetyl-transferase) or neomycin resistance, and the polyadenylation signal of the bovine growth hormone. Correct homologous recombination will result in a modified BRCA2 gene that can transcribe a bicistronic mRNA. Ribosomes can enter the bicistronic mRNA either at the 5' end to translate the gene of BRCA2 or at the ECMV IRES to translate the antibiotic resistance marker. Since the selection gene lacks its own promoter, the expression of the resistance marker genes is only activated from the BRCA2 promoter following correct homologous integration.

We had performed many rounds of transfection. However, there are only very few positive clones that were selected out when very low concentration of Neomycin or Puromycin were used. Out of the clones selected out, we were unable to identify any positive clones by PCR and Southern Blotting.

D. Generation of the second set of promoterless knock out constructs and transfection into hTERT immortalized normal mammary epithelial cells to screen for BRCA2$^{+/-}$ cells.

Since we did not obtain any positive recombination in many experiments, the locus we selected could be the culprits. It has been reported that the frequency of homologous recombination can be influenced by the position of targeting locus. Therefore, we have chosen another region of BRCA2 gene to design another set of knock out construct. As shown in Fig. 4, a short 5' arm was generated from part of intron 2 and exon 3 by PCR with ClaI and XhoI restriction site incorporated. A stop codon was added to stop the translation of BRCA2 just before XhoI restriction site. A long 3' arm was generated from BRCA2 gene covering part of exon 3, intron 3, exon 4, intron 4, exon 5, intron 5, exon 6 and part intron 7 (BglII and BamHI restriction site will be added). The resulting fragment was cloned into pKO-NYKV sequentially. One set of positive control plasmids were constructed by generating a longer 5' arm by PCR as illustrated in Fig. 4.

We had performed many rounds of transfection. We encountered the same problem as we encountered using exon 11 promoterless targeting vectors. There are very few positive clones that can be selected out when very low concentration of Neomycin or

Puromycin were used. Out of the clones selected out, we were unable to identify any positive clones by PCR and Southern Blotting.

E. Assess the feasibility to use GFP and FACS sorter to select positive clones. One problem we encountered using promoterless construct is that we can only obtain very few clones after selecting a very large number of transfected cells. The number of cells we can select is the limiting factor. Since selection can take up to two weeks using low concentration of antibiotics such as G418. Therefore, we would like to try to select the cells using GFP and FACS sorting. GFP have been widely used for FACS cell sorting. Theoretically, a promoterless GFP can be constructed to replace promoterless Neo and Puro genes for efficient and quick selection of correctly recombinated cells. To test the feasibility of this approach, we transfected normal mammary epithelial cells, 76N or its immortal derivative 76NTert cells with pEGFP to try to sort out GFP positive cells by FACS sorting. However, we found that the normal mammary epithelial cells we tested are unable to sustain the process of FACS sorting. Therefore, GFP is not feasible to be used as a promoterless selection marker.

F. Generation of promoterless knock out constructs using magnetic beads selection methods. Instead of FACS sorting, another approach to select cells using cell surface markers is to use antibody coated magnetic beads. The magnetic beads approach is much milder than FACS sorting. To test the feasibility, we transfected hTert immortalized mammary epithelial cells with pCMV-CD19+pcDNA-β-gal. The cells were harvested 48 hours after transfection and subjected to selection using magnetic beads (Dynabeads® CD19, Dynal Biotech). After selection the beads were detached from the cells using DETACHaBEAD® CD19 (Dynabeads® CD19, Dynal Biotech). We found that the positively transfected cells can be efficiently selected and the selected cells are health and proliferating. Therefore, we designed two set of knock-out constructs using CD19 and Hook-2 as the promoterless selection marker as shown in Fig. 5 and Fig. 6 to target both exon 2 and exon 11. We are in the process of generating these two sets of constructs.

G. The RNAi approach. Our RNAi strategy has been successful. We already obtained a number of clones with BRCA2 expression level reduced to different levels, which are suitable for assessing their susceptibility to radiation induced transformation as show in Fig. 7. However, RNAi strategy does not ideally mimic the in vivo situation. We plan to use it as a backup strategy if our modified strategy using magnetic beads selection method is proven to be unsuccessful.

We will treat these cells with γ-radiation and asses the susceptibility of these cells to radiation induced transformation. Although these cell lines will not be same as the BRCA2 heterozygous cell lines, we should be able to assess the effect of reduced BRCA2 expression on the susceptibility of these cells to radiation induced transformation.

Task 4. Assess susceptibility of BRCA2$^{+/-}$ human mammary epithelial cells to spontaneous immortalization by sub-culturing. (months 13-20)

Although we can not detect the positive recombined colonies, theoretically, there exist some correctly recombinated colonies existed in the pool populations. If knockout of one allele of BRCA2 render the cells susceptible to spontaneous immortalization of normal mammary epithelial cells, long term culture of the 76N cells that were transfected with the BRCA2 knockout construct should select out the spontaneous immortalized cells. For this purpose, we cultured the pool population of the transfected cell for 7 month (about 9 passages), however, no spontaneous immortalization was observed.

Task 5. Characterize the spontaneous immortalized cells. (months 20-26)

Since we did not yet obtain spontaneous immortalized cells, this task is no long relevant.

Task 6. Assess susceptibility of BRCA2$^{+/-}$ human mammary epithelial cells to radiation induced immortalization. (months 13-24)

Since we have not obtained BRCA2$^{+/-}$ human mammary epithelial cells, we still have not carried out this task yet.

KEY RESEARCH ACCOMPLISHMENTS

- Tested two promoterless constructs with puromycin and neomycin resistance gene respectively.
- Generated and tested another set of knock out construct to target the exon 2 region of BRCA2.
- Established stable BRCA2 knock down cell lines using BRCA2 RNAis
- Test the feasibility to use GFP and FACS sorting as selection method
- Test the feasibility to use cell surface marker such as CD19 and Hook-2 for selection using magnetic beads.
- Designed two set of knock out constructs to target exon 2 or exon 11 using cell surface marker as promoterless selection marker.

REPORTABLE OUTCOMES

No reportable outcomes in this year.

CONCLUSIONS

In the approved Statement of Work, we proposed six tasks for the first two years. The task 3 is: Transfect the knock out construct into normal mammary epithelial cells and screen for clones with one allele of BRCA2 disrupted. This task turned out to be much more difficult than we have anticipated. Thus far we have not yet obtained clones with one allele of BRCA2 disrupted even after numerous attempts. Our experience of failure is in accord with the common notion that creating a knockout cell line is a time consuming, labor-intensive process with an uncertain success rate. However, to address an important problem such as the one proposed here, persistent effort is warranted. We are currently in the process of more attempts with modified strategies (Table 1).

Table 1. Strategies attempted and planned

Homologous recombination	Exon 11 positive and negative selection	attempted	Failed	Original proposed strategy. Fig. 2a, 2b
Homologous recombination	Exon 11 promoterless approach	attempted	Failed	Fig. 3a, 3b
Homologous recombination	Exon 2 promoterless selection	attempted	Failed	Fig. 4
Homologous recombination	Promoterless GFP FACS sorting selection	Preliminary experiment performed	Not feasible	
Homologous recombination	Promoterless Hook-2 Magnetic selection	planned	To be performed	Fig. 5a, 5b
Homologous recombination	Promoterless CD19 Magnetic selection	planned	To be performed	Fig. 6a, 6b
RNAi	Six RNAi constructs generated	Attempted.	Many cell clones obtained Some of them are suitable for further experiment	Fig. 7 Less ideal. Unable to mimic the in vivo situation, To be used as back up strategy

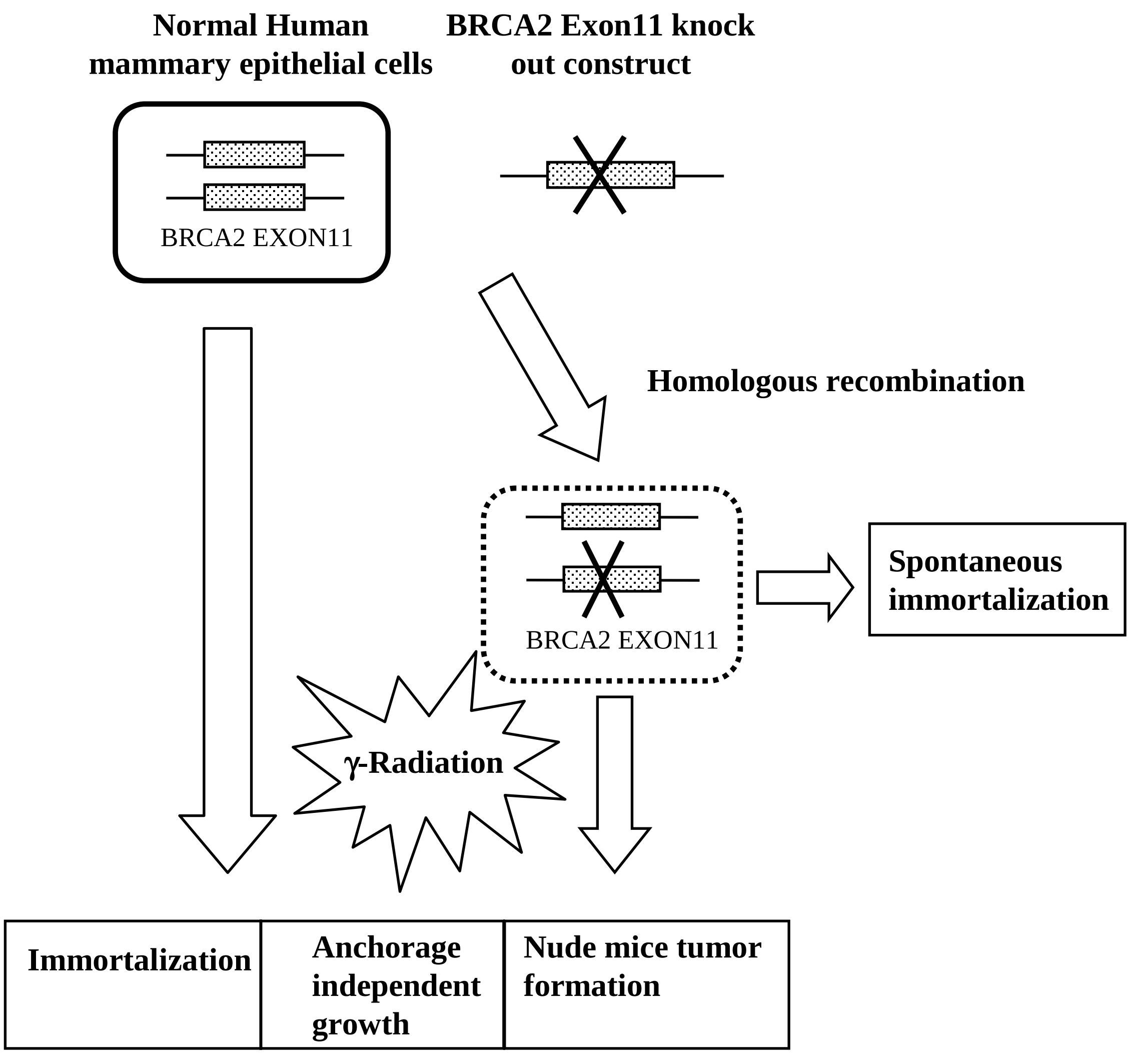

Fig. 1. Generation of a BRCA2 heterozygous mammary epithelial cell strain and comparing the susceptibility of the BRCA2$^{+/-}$ cells and its parental cells to radiation-induced transformation.

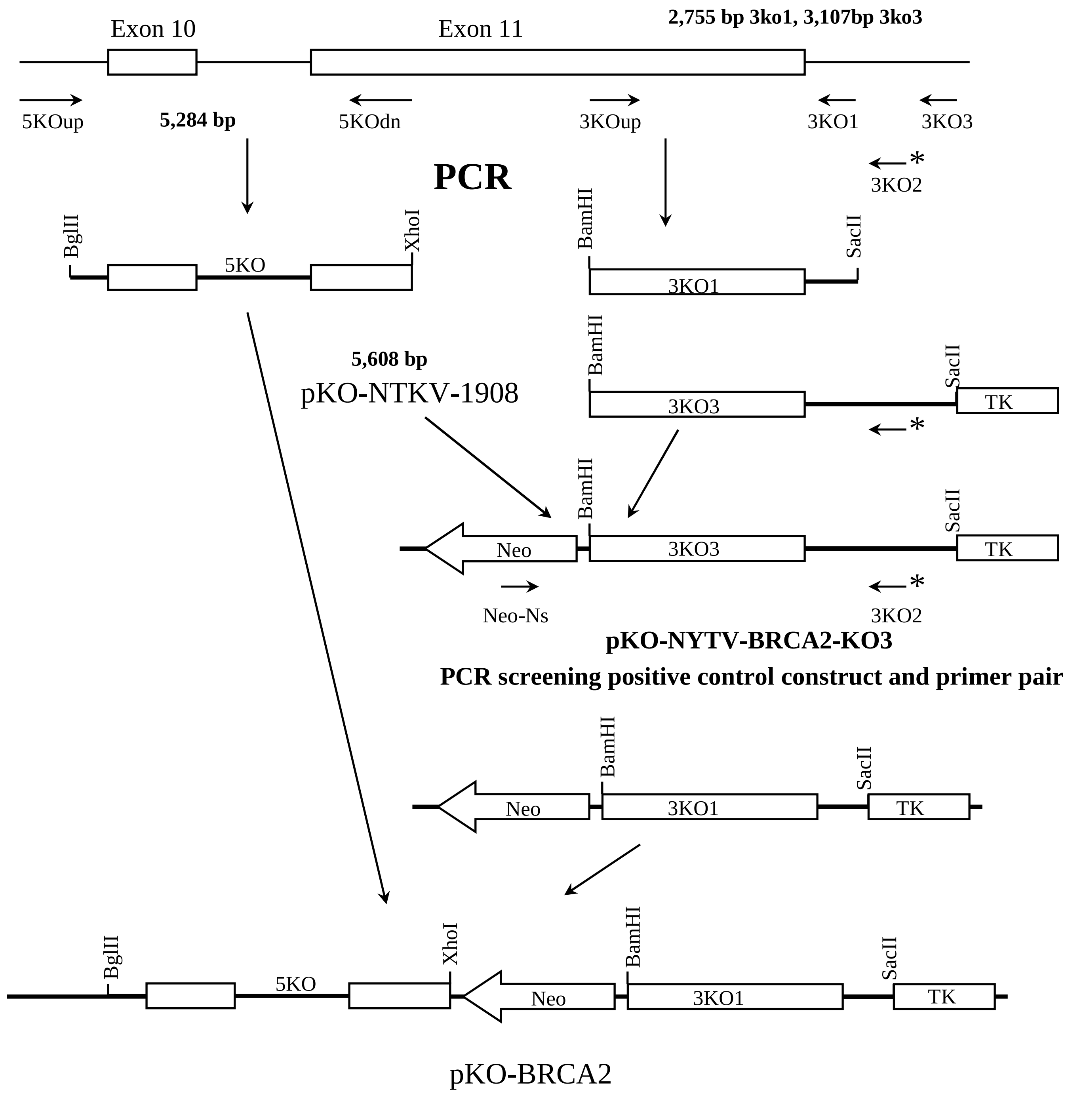

Fig. 2a. Schematic of the positive negative selection BRCA2 knockout vector and the positive control plasmid design.

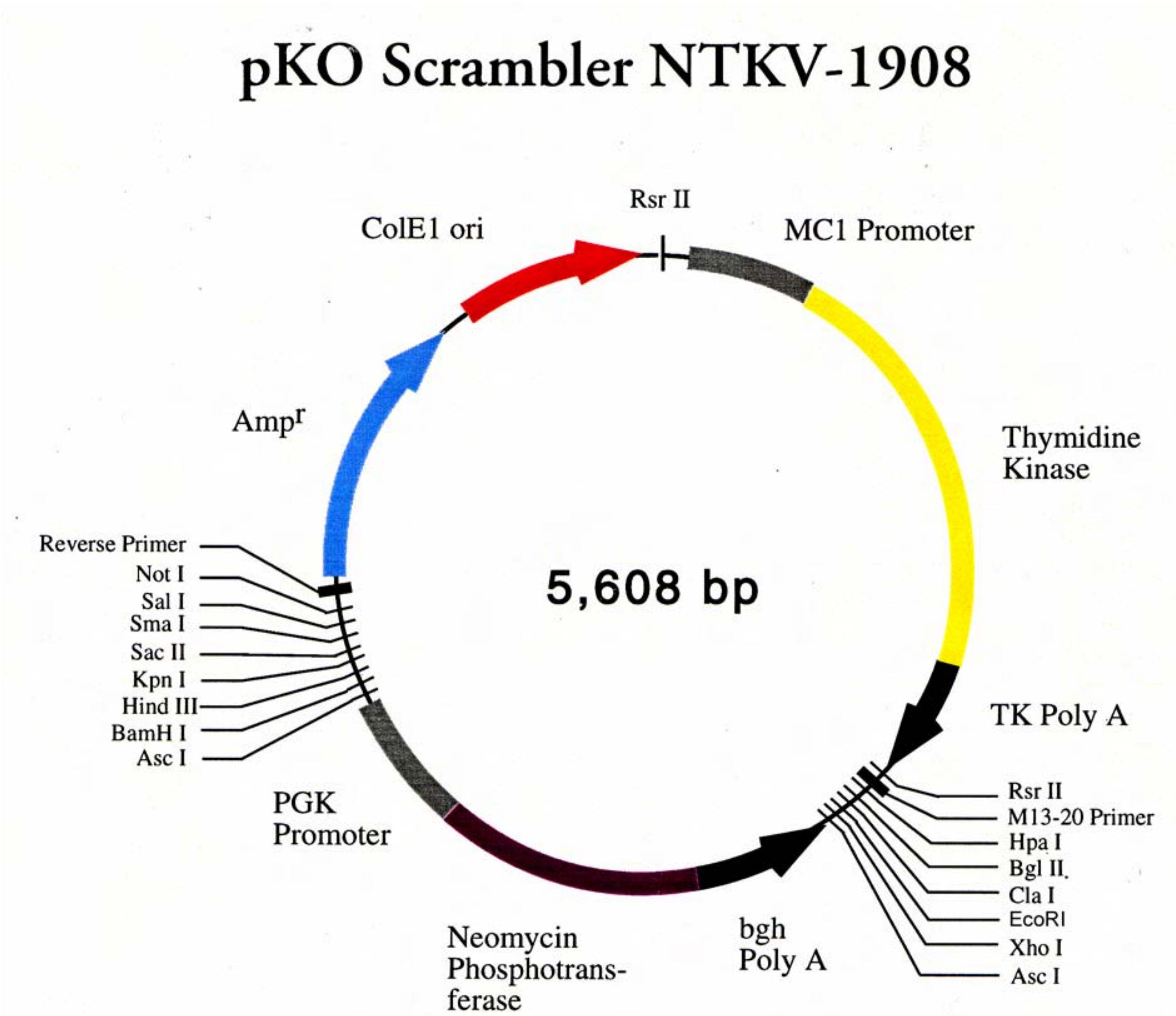

Fig. 2b. Schematic of pKO NTKV-1908.

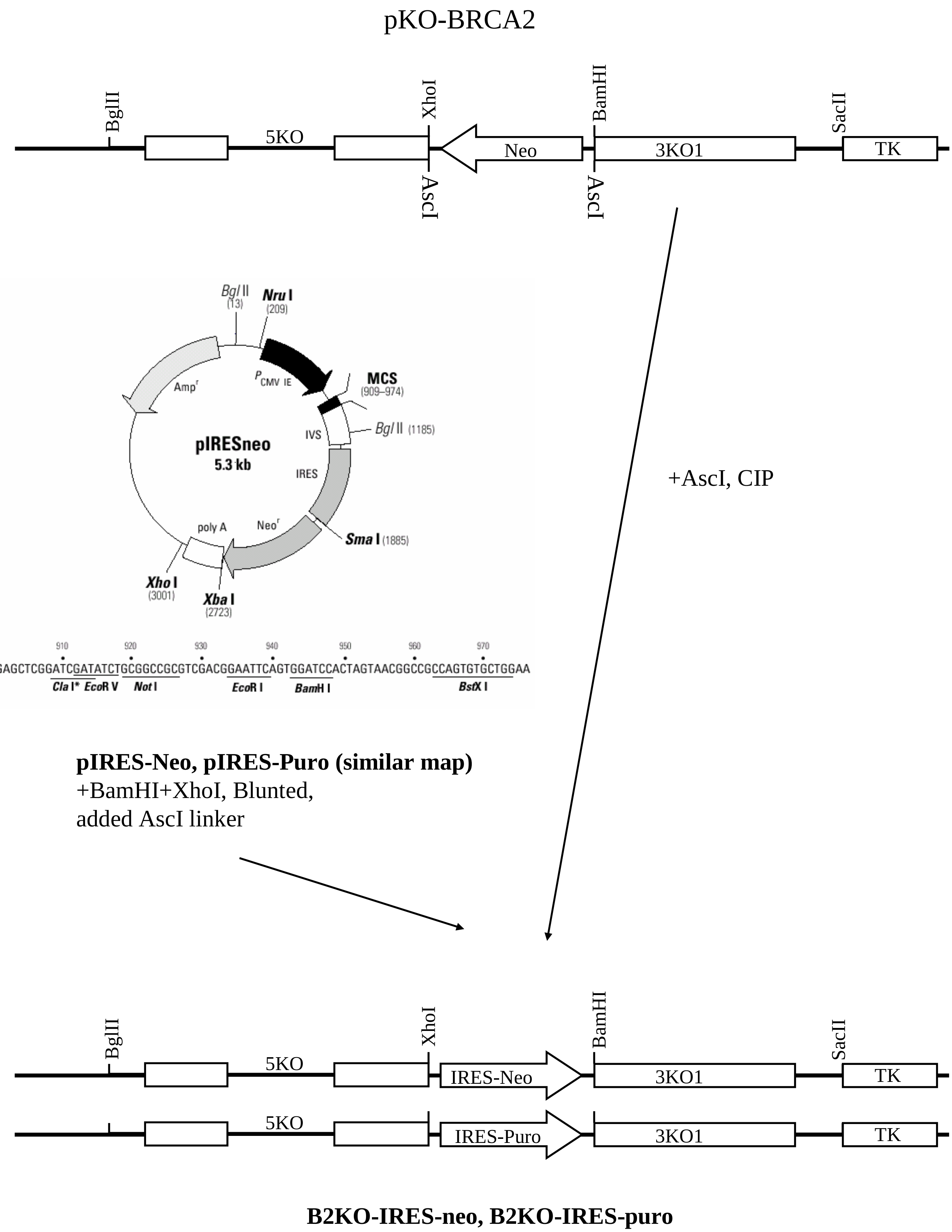

Fig. 3a. Schematic of the promoterless BRCA2 knockout design.

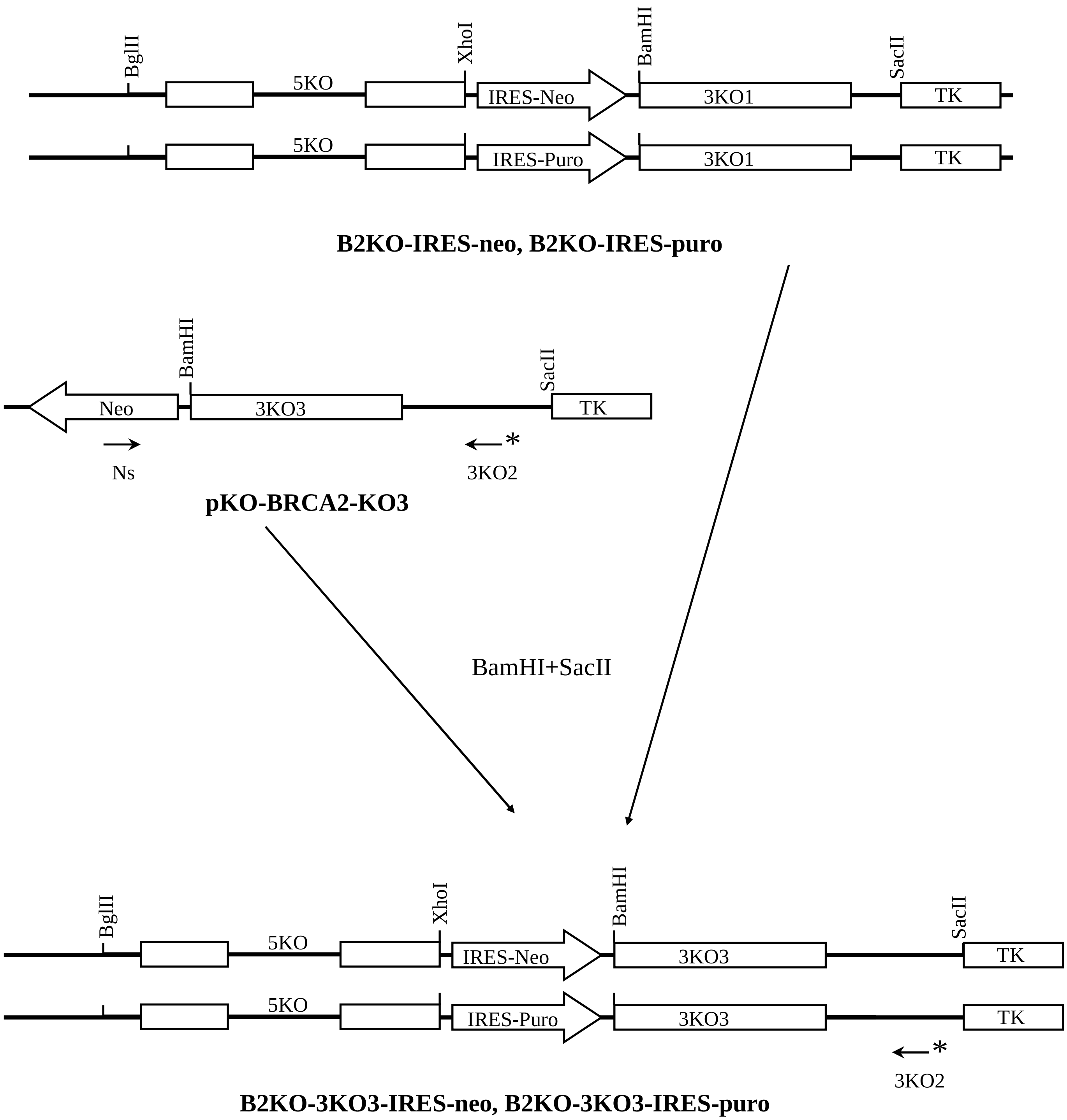

Fig. 3b. Schematic of the positive control plasmids design for the promoterless BRCA2 knockout vector.

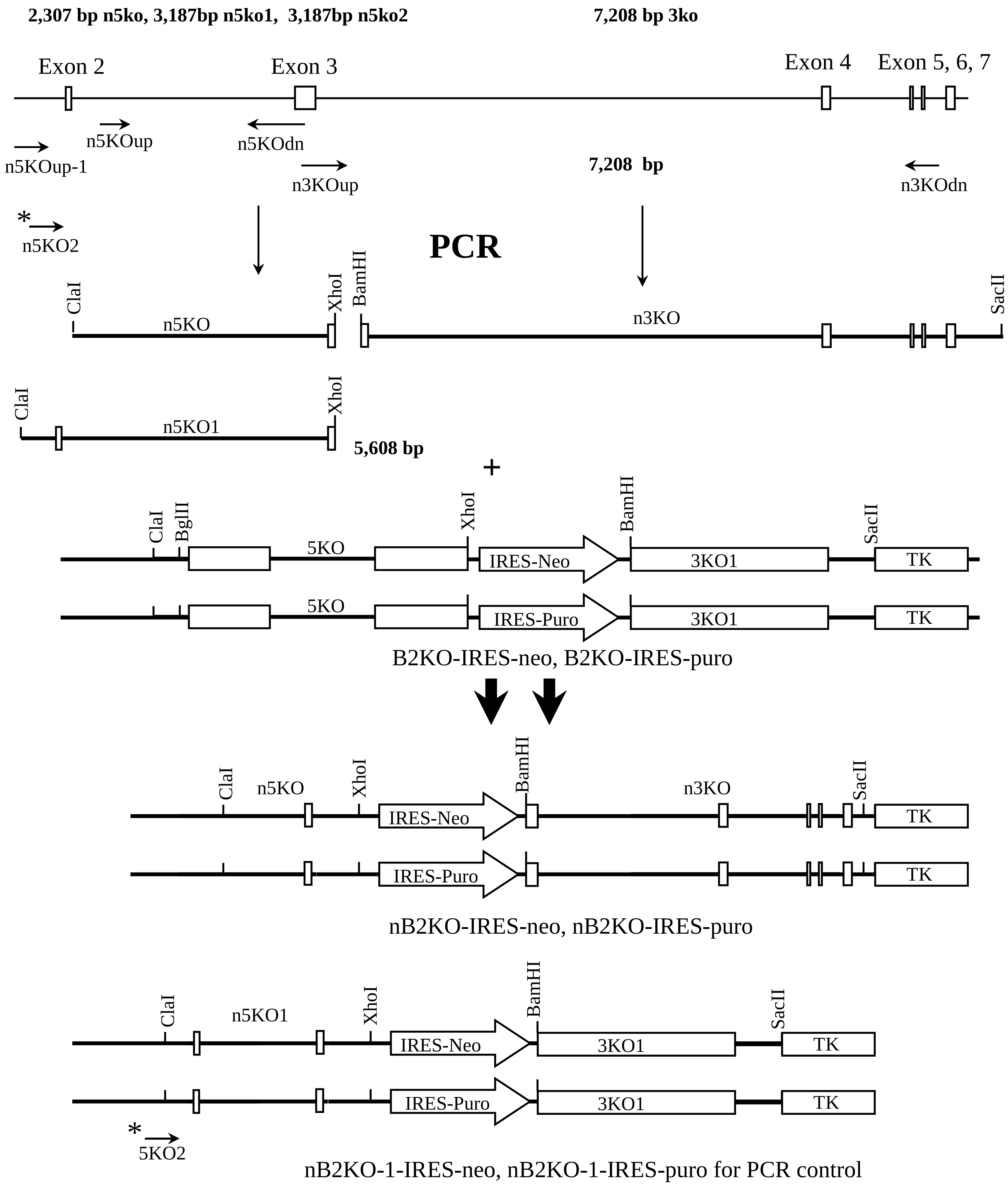

Fig. 4. Schematic of exon 2 promoterless BRCA2 knockout vector and its positive control plasmids design.

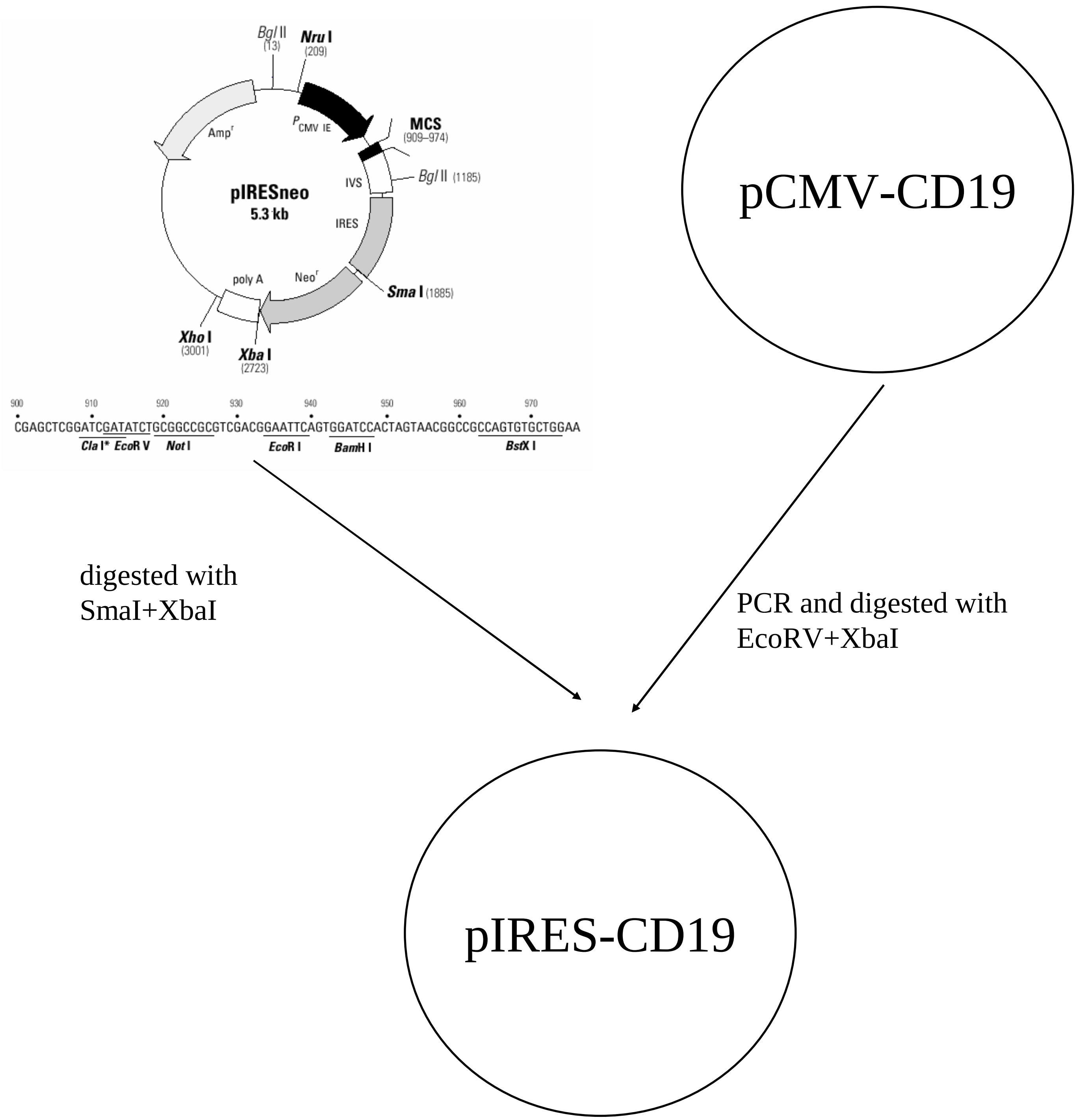

Fig. 5a. Schematic of pIRES-CD19.

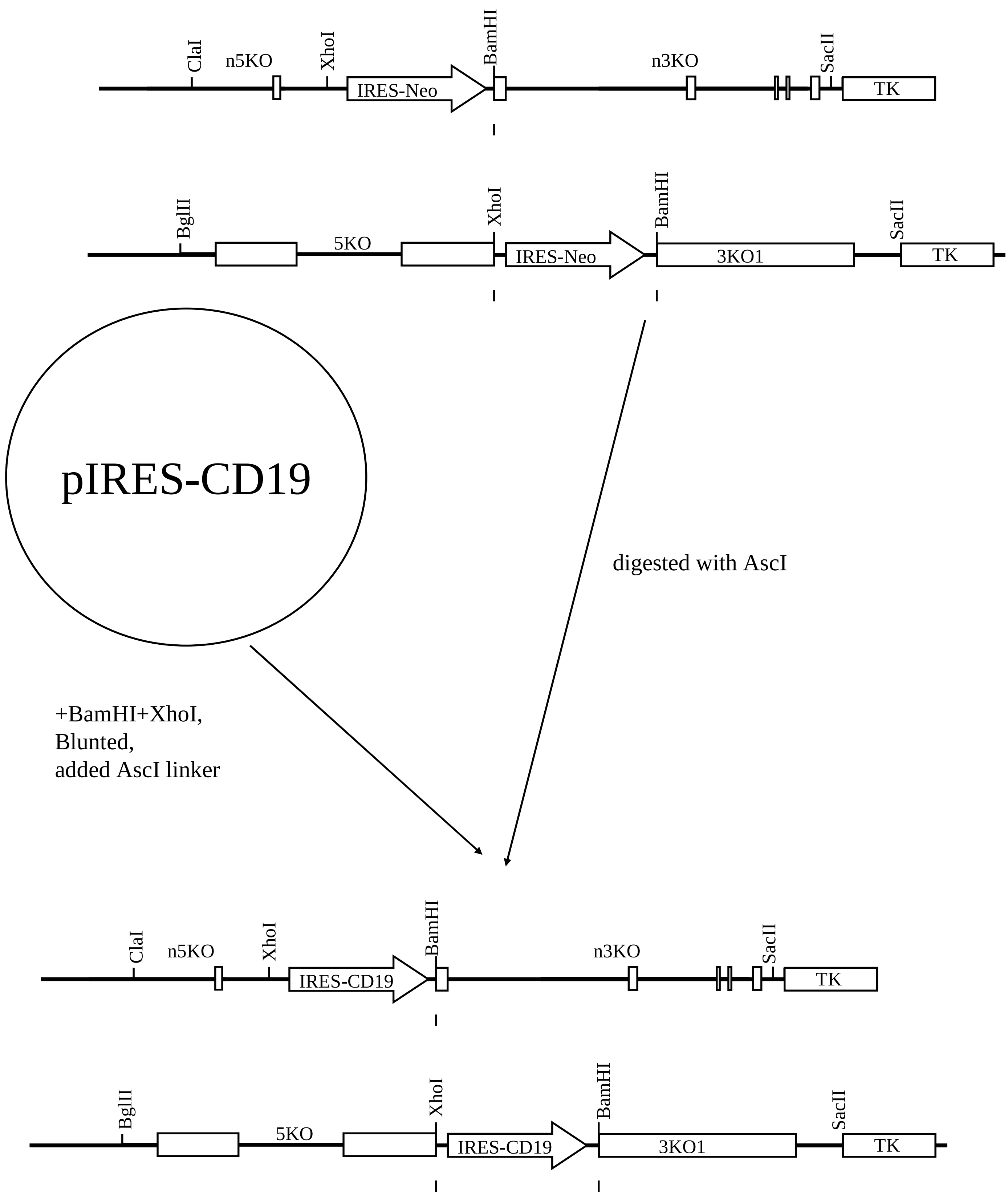

Fig. 5b. Schematic of exon 2 and exon 11 promoterless BRCA2 knockout vector with CD19 for magnetic selection.

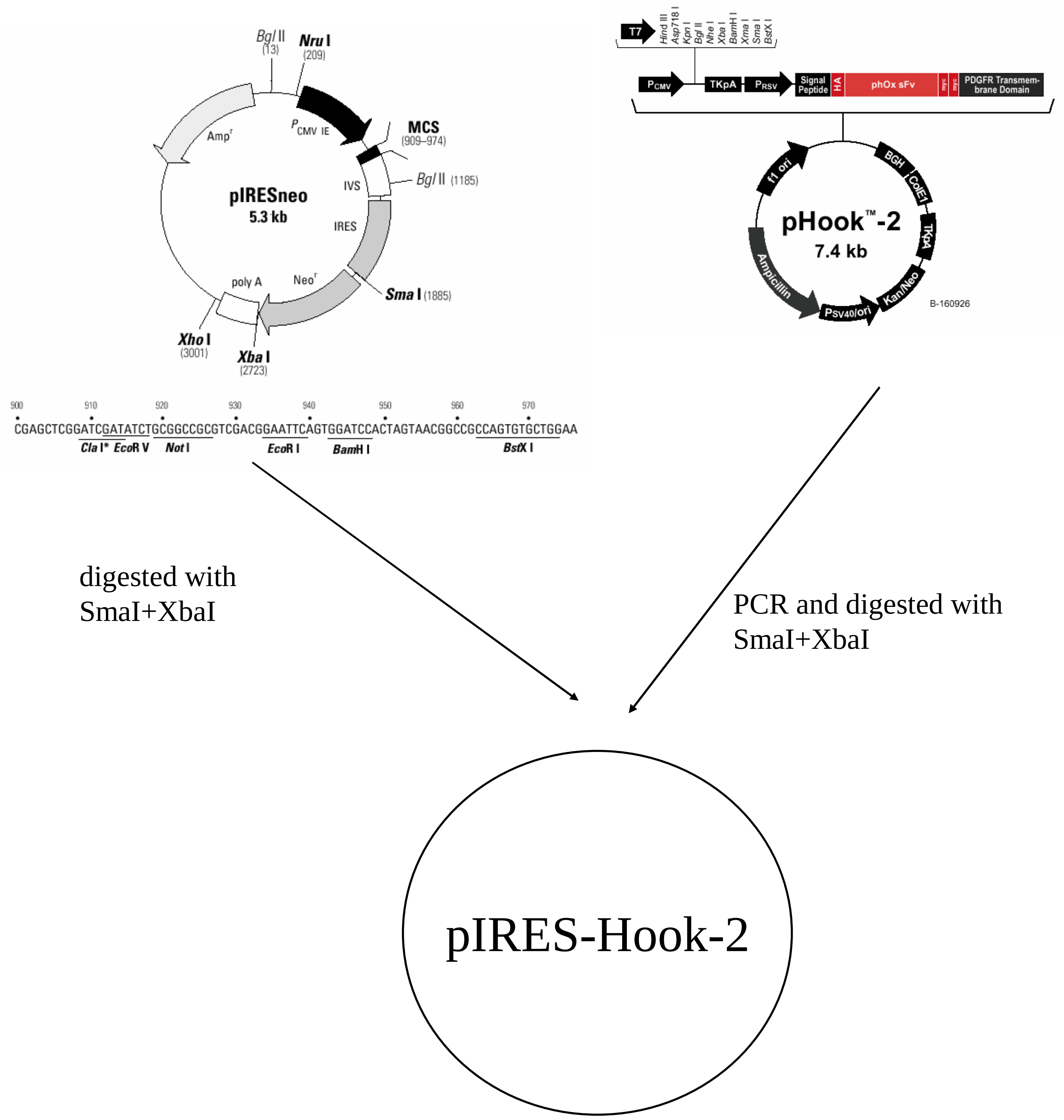

Fig. 6a. Schematic of pIRES-Hook-2.

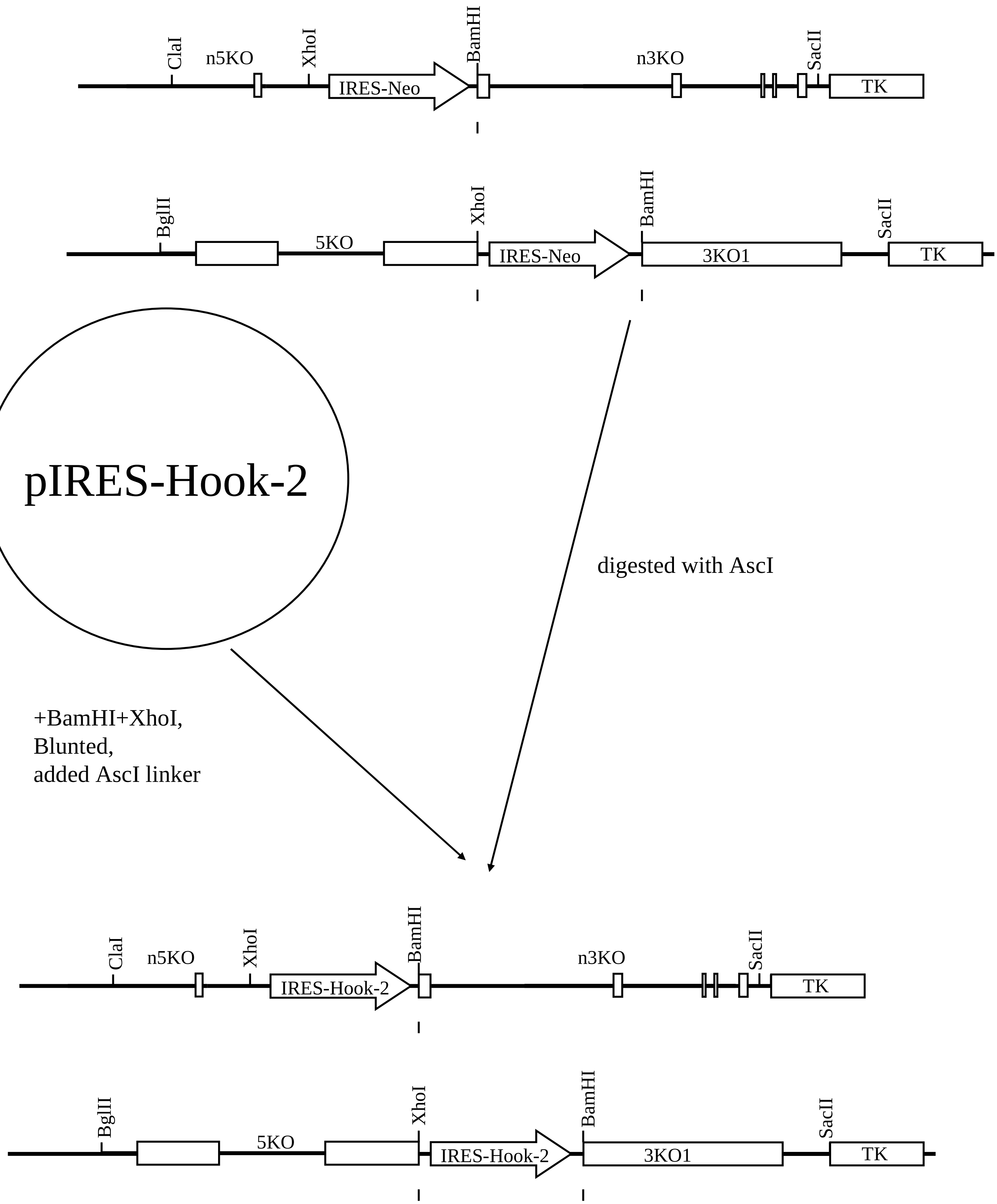

Fig. 6b. Schematic of exon 2 and exon 11 promoterless BRCA2 knockout vector with Hook-2 for magnetic selection.

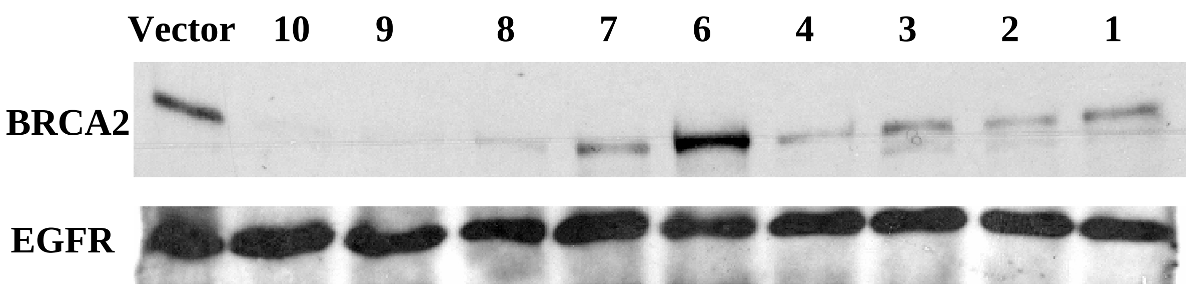

Fig. 7. BRCA2 western blotting analysis of stable 76Tert cell clones that were infected with BRCA2 RNAi. 76Tert cells was infected with vector or pSuper-retro-BRCA2-444. The cells was selected for about two weeks. Cell clones were picked and cultured. The cells were harvested in Sample Buffer and fractionated on a 5% SDS-PAGE gel. Western Blotting was performed using anti-BRCA2 antibody Ab1 (oncogene science) and anti-EGFR (use as loading control).

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APPENDICES: No.